Remarks

Claims 1-32 are pending in the application. Claims 1-11, 13, and 16-32 are canceled without prejudice to the filing of a continuing application. Claims 12, 14 and 15 have been amended. New claims 33-37 have been added. Claims 12, 14, 15 and 33-37 are now pending.

Support for amended claim 12 is found in Example 1, pg. 17 in para. 0064. Support for amended claim 14 is found on pg. 3, para. 0009, pg. 6, paras. 0010-0011 and on pg. 13, para. 50 of the specification. Support for amended claim 15 is found on pg. 12, para. 0047 of the specification. Support for new claims 33 and 35 is found on pg. 12, para. 0047 of the specification and in original claims 4 and 14. Support for new claim 34 is found on pg. 13, para. 0050 of the specification and in original claim 14. Support for new claims 36-37 is found on pg. 18, para. 0065. No new matter has been added.

Applicants request reconsideration of the claims based on the above changes and the following remarks.

Objection to the Specification

The Examiner has objected to the incorporation by reference of the list of publications found on pgs. 30-37, or otherwise cited, in the specification. M.P.E.P. 608.01(p) states that non-essential subject matter may be incorporated by reference to non-patent publications for the purpose of indicating the background of the invention or illustrating the state of the art. The incorporation by reference of these non-patent publications is therefore considered proper.

The incorporation by reference of those non-patent publications found on pgs. 30-37, or otherwise cited, in the specification is not meant to indicate that any of the referenced publications are material to the patentability of the solicited claims. Documents considered material to the patentability of the solicited claims will be submitted on an Information Disclosure Statement.

Objection to Claims 14 and 18

Claims 14 and 18 are objected to because of spelling errors. Claim 18 has been canceled, and the objection is therefore moot. Claim 14 has been amended to correct "CBS+T" to "CB8+T".

Claim Rejections Under 35 U.S.C. § 112

Claims 1-2, 14-18, 20 and 25 are rejected under 35 U.S.C. § 112, 1st paragraph as allegedly failing to comply with the enablement requirement. Claims 1-2, 16-18, 20 and 25 have been canceled, and the rejection is moot as to these claims. The rejection will therefore be discussed with regard to claims 12, 14 and 15 as amended, and newly added claims 33-37.

A specification is enabling if it teaches one skilled in the art how to make and use the claimed invention without undue experimentation.

Claims 12, 14 and 15 and 33-37 are directed to compositions for expanding expression of virus-specific CB8+T cells. The claimed compositions comprise an antigen-presenting cell which is pulsed with inactivated HIV. Dependent claim 12 specifies that the inactivated HIV used to make the claimed composition is an autologous virus. Dependent claim 15 specifies that the virus-specific CB8+T cells kill the HIV-infected cells. Dependent claims 33-35 define the antigen-presenting cells as, respectively, dendritic cells, autologous dendritic cells, or monocyte-derived dendritic cells. The claimed compositions are used to treat HIV. Dependent claims 36-37 specify that the HIV used to make the claimed composition is inactivated chemically (claim 36), specifically by 2,2'-dithiopyridine or aldrithiol-2 (claim 37).

The Official Action contends that the specification, at the time of filing, would not have taught one skilled in the art how make or use the claimed compositions. The Official Action also appears to doubt that the claimed compositions would actually treat HIV. Although this latter point more closely resembles a utility rejection, the Applicants will address the issue here.

According to the Official Action, it was known in the art that retroviral infections were refractory to treatment, in particular due to the extensive genomic diversity and high mutation rate of HIV, the mode of HIV transmission within a host, latent virus reserves, the ability to cross the blood-brain barrier and the variation in the pathology of HIV infection among individuals. The Official Action also notes that, although the body produces anti-HIV antibodies upon infection with the virus, these antibodies do not halt the spread of HIV. The Official Action further cites to Fahey et al. and Fox to show that clinical trials of immunologically-based therapies have not been successful in treating HIV infection. Applicants note that Fahey et al. was published nine years, and Fox was published 7 years, before the filing date of this application. One skilled in the art would therefore likely not consider that these references represent the state of the art at the time this application was filed. Regardless, the Applicants

have presented sufficient data in this specification to show that the presently claimed compositions are enabled.

As discussed above, the claims as amended are directed to compositions for expanding CB8+T cells, comprising antigen-presenting cells that have been pulsed with inactivated HIV. The working examples disclosed in this specification show that CB8+T cells are indeed expanded by the claimed compositions. See, *e.g.*, Example 1 (pgs. 17-21) and Fig. 2, which show the proliferation of patient T cells following stimulation with HIV-pulsed autologous dendritic cells. The data presented in Example 1 also show that HIV-infected B-LCL cells were killed, and that the killing of these cells was carried out by CB8+T cells (see pg. 19, para. 0066). The antiviral activity of CB8+T cells expanded by HIV-pulsed dendritic cells (with or without protease inhibitors) was achieved equally in untreated and HAART-treated HIV patients, regardless of their CD4 cell counts or viral load. The specification therefore teaches one skilled in the art how to make and use the claimed compositions, and that the compositions do in fact cause the expansion of CB8+T cells that kill HIV-infected cells.

The Official Action notes a consistent failure of HIV patients to mount an effective immune response with prior art anti-HIV therapies. However, the solicited claims are not directed to the treatment of HIV, but only to compositions which expand CB8+T cells. The Applicants' specification clearly teaches how to make the claimed compositions, and use them to successfully expand HIV-specific CB8+T cells. The concerns of the Official Action regarding the obstacles faced in treating HIV, and the failure of previous HIV treatments, are therefore not relevant to the solicited claims. Nevertheless, as is shown by the data presented in the specification, the claimed compositions overcome the shortcomings of previous HIV therapies. As stated in the specification, at pg. 21 paras. 0070-0071:

> The aforementioned above referenced data and results as described in Example 1 provide the first evidence that a high frequency of PMBC harboring HIV can be eradicated in vitro by cultured patient T cells expanded with inactivated-virus-pulsed autologous DC. This potent antiviral activity of patient T cells stimulated with virus-pulsed DC is CD8 dependent and (is) independent of the patient's disease stage and treatment status . . .The viral clearance obtained in vitro with autologous T cells expanded by inactivated-virus-pulsed DC opens the possibility of an in vivo restoration of anti-HIV immunity.

The Applicants provide a manuscript from their laboratory (Lu et al., "Therapeutic dendritic-cell vaccine against chronic HIV-1 infection"; copy enclosed) that discloses the

successful *in vivo* treatment of HIV infection in humans by administration of the claimed compositions. In the manuscript, data is presented which shows that a therapeutic composition made from patient-matched dendritic cells loaded with aldrithiol-2-inactivated HIV-1 expanded CB8+T cells, *in the absence of significant neutralizing antibodies*, significantly reduced viral loads in chronically-infected HIV patients.

According to the Lu et al. manuscript, blood CD4+ cell counts in the treated patients increased significantly from week 4 post-vaccination, but returned progressively to baseline level after week 16, whereas CB8+T cell counts remained unchanged throughout the study (see Fig. 1a of the manuscript). Blood HIV-1 cellular viral load ("CVL") and plasma viral load ("PVL") levels decreased by 40% ($p<0.001$) and 85% ($p<0.001$), respectively, over the 16 weeks post-vaccination, and these levels remained stable for up to 360 days (the extent of the study).

These data show that the obstacles to successful *in vivo* treatment of HIV in humans noted in the Official Action have been overcome by the claimed compositions, and the failure of previous anti-HIV therapies reported by Fahey et al. and Fox are a thing of the past. As noted in the Applicants' specification at para 0010 on pgs. 22-23 (emphasis added):

> Although the in vivo evolving HIV-1 variants that evade the antiviral immunity developed during early infection have been know for many years, the reason that the infected host fails to mount de novo mutant-virus-specific immunity remains unknown. In a chronically HIV-infected individual (i.e., one in whom the virus has already been disseminated into lymph nodes), viral replication is directly linked to local activation of lymphoid tissues characterized by huge in situ expression and release of cytokines. Certain components of these lymphoid cytokines . . . are known to interfere with generation of immature DC, and others . . . provoke DC maturation. Since supermatured DC lose their ability to process and present viral antigens (Fig. 4), it is conceivable that supermatured DC in immune-activated lymphoid tissues could not exert their APC function to process and present the evolving mutant antigens of viral variants. Such paralyzed DC in situ, in fact, could thus provide the prerequisite for establishing chronic HIV infection. ***However, our data demonstrate that such a defect in the generation of functional DC in HIV infected patients can be overcome by DC-based vaccines generated in vitro from peripheral blood monocytes taken from infected patients.***

Thus, compositions which expand CB8+T cells can successfully be made and used by following the teachings of this specification. The expanded CB8+T cells resulting from the claimed compositions can kill HIV-infected cells, and in fact the claimed compositions have been used to successfully treat chronically-infected HIV patients, overcoming long-standing

obstacles to HIV treatment. No undue experimentation is needed to practice the solicited claims, as the full teaching required to make and use the claimed compositions is contained within the Applicants' specification. Claims 12, 14-15 and 33-37 are therefore enabled by the Applicants' specification, and the rejection of these claims under 35 U.S.C. 112, first paragraph should be withdrawn.

Claim Rejections under 35 U.S.C. § 102 and 102(a) and (b)

Claims 1, 4-5 and 11 are rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Frank et al. Claims 1, 4-5 and 11 have been canceled, and therefore this rejection is moot.

Claims 12, 14, 15 and 33-37 are directed to compositions for expanding CB8+T cells, comprising antigen-presenting cells that have been pulsed with inactivated *human* immunodeficiency virus (HIV). Frank et al. discloses inactivated *simian* immunodeficiency (SIV) virus in immature dendritic cells. As Frank et al. does not disclose every element of claims 12, 14, 15 and 33-37, these claims cannot be anticipated by this Frank et al.

Claims 14 and 17 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Grovit-Ferbas et al. Claim 17 has been canceled, and the rejection is moot as to this claim. The rejection will be discussed with regard to claim 14 and its dependent claims 12, 15 and 33-37.

Grovit-Ferbas et al. disclose a dendritic cell which contains heat-inactivated HIV. This dendritic cell was able to induce a cell-mediated recall response *in vitro*, as measured by the capacity to induce gamma interferon production in the PBMC from three HIV patients, none of whom had a detectable viral load. No data is presented in Grovit-Ferbas et al. which showed that the dendritic cell of Grovit-Ferbas et al. expanded CB8+T cells or was capable of inducing the CB8+T cells to kill HIV-infected cells. In fact, according to Grovit-Ferbas et al., pg. 5808, 2nd column:

> heat-inactivated preparations also elicited memory cell-mediated immune responses in vitro. Although it is not clear which cell subset produced IFN-γ in response to our vaccine preparation, it is likely that the cytokine was secreted by CD4 cells, since the DC were given an exogenous (antigen) for processing.

Claims 12, 14-15 and 33-37 are directed to compositions for expanding expression of virus-specific CB8+T cells. At best, the dendritic cells of Grovit-Ferbas et al. elicits IFN-γ

production from CD4 cells, and there is no evidence that the dendritic cells can expand CB8+T cells. Thus, Grovit-Ferbas et al. does not anticipate the solicited claims.

In particular, dependent claim 12 specifies that the inactivated HIV used to prepare the claimed composition is an autologous HIV. Grovit-Ferbas et al. did not use an autologous HIV to prepare their vaccine, but rather used HIV_{sx}, which is a primary virus isolate. See Grovit-Ferbas et al. pg. 5803, 1st col. Thus, Grovit-Ferbas et al. cannot anticipate claim 12.

Dependent claim 15 specifies that the virus-specific CB8+T cells kill the HIV-infected cells. There is no evidence presented in Grovit-Ferbas et al. that the disclosed dendritic cells can expand CB8+T cells that kill HIV-infected cells. Thus, Grovit-Ferbas et al. cannot anticipate claim 15.

Dependent claims 36-37 specify that the HIV used to make the claimed composition is inactivated chemically (claim 36), such as by 2,2'-dithiopyridine or aldrithiol-2 (claim 37). The inactivated HIV used to prepare the Grovit-Ferbas et al. dendritic cells was heat-inactivated. Thus, Grovit-Ferbas et al. cannot anticipate claims 36-37.

Based on the foregoing, the Applicants request that the 35 U.S.C. § 102(b) rejection be withdrawn.

Claim Rejections under 35 U.S.C. § 103(a)

Claims 1, 2, 11-12, 14-18 and 20 are rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by Frank et al. Claims 1, 2, 11, 13, 16-18 and 20 have been canceled, and the rejection is moot as to these claims. The rejection will be discussed with respect to claim 14 and dependent claims 12, 15 and 33-37.

Frank et al. discloses a composition consisting of inactivated *simian* immunodeficiency (SIV) virus in immature dendritic cells. The solicited claims are directed to compositions for expanding CB8+T cells, comprising antigen-presenting cells that have been pulsed with inactivated *human* immunodeficiency virus. The Official Action contends that it would have been obvious to one skilled in the art to make compositions from inactivated HIV, as claimed herein, from the teaching in Frank et al. of compositions made from SIV.

A reference must suggest to one skilled in the art that the claimed invention can be made and successfully practiced to render a claim obvious. Here, Frank simply presents data that human and macaque dendritic cells interact similarly with SIV. Although HIV and SIV share

similar structural and immunologic properties, the data presented in Frank et al. are not necessarily predictive of how dendritic cells would interact with HIV. Moreover, Frank et al. does not present any data showing that dendritic cells containing inactivated SIV can elicit a protective immune response. In the absence of such data, one skilled in the art would therefore likely not assume that dendritic cells pulsed with inactivated HIV could elicit a protective immune response. See, *e.g.*, Grovit-Ferbas et al. (cited by the Examiner) at pg. 5802, 2nd col., which states that "[e]arly efforts to model a killed HIV-1 vaccine using SIV . . . were unsuccessful" (emphasis added).

At best, therefore, one skilled in the art might consider the production of a composition comprising a dendritic cell pulsed with inactivated HIV "obvious to try." Obvious to try is not the standard of patentability. Moreover, Frank et al. does not provide the required reasonable expectation that the claimed compositions could be successfully made and used. The 35 U.S.C. § 103(a) rejection should therefore be withdrawn.

Finally, Applicants submit Lu et al. (Oct. 2001), *J. Virol.* 75: 8949-8956 (copy enclosed), which is an article by the Applicants disclosing the claimed compositions and their use in expanding HIV-specific CB8+T cells. The Lu et al. article, which was published less than one year before the filing date of this application, was nevertheless published prior to the March 2002 publication date of Frank et al. Thus, Lu et al. shows that the Applicants had reduced the invention to practice prior to the publication of Frank et al. Frank et al. therefore cannot be prior art to the solicited claims, and cannot support a rejection of these claims under 35 U.S.C. § 103(a).

Conclusion

In view of the foregoing, the Applicants respectfully submit the Application is now in condition for allowance, which is respectfully requested.

Sincerely,

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PC/JEB: pm
215.656.3320

Encls.:

Lu et al., "Therapeutic dendritic-cell vaccine against chronic HIV-1 infection". Lu et al. (Oct. 2001), *J. Virol.* 75: 8949-8956.

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<arttitle> Therapeutic dendritic-cell vaccine against chronic HIV-1 infection

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<abs> Therapeutic vaccines made of chemically inactivated human or simian immunodeficiency virus (HIV or SIV)-loaded dendritic cells (DCs) have recently been demonstrated to induce protective antiviral immunity in infected animals [1-3]. Here, we show in 18 chronically HIV-1-infected patients without antiviral therapy that a therapeutic vaccine made of aldrithiol-2-inactivated HIV-1-loaded patient-matched DCs promptly boosted specific $CD4^+$ T-helper type 1 (Th1) cells and $CD8^+$ ($perforin^+$) effectors in the absence of significant neutralizing antibodies. One year after the vaccination (made of three immunizations at two-week intervals), patients exhibited a plasma viral load decrease of 85%. Frequencies of HIV-1-specifc interleukine-2- and interferon-γ-expressing $CD4^+$ Th1-cell and HIV-1 gag-specific perforin-expressing $CD8^+$ effector responses were positively correlated with levels of viral-load suppression, suggesting that a robust virus-specific $CD4^+$ Th1 response is required for inducing and maintaining virus-specific $CD8^+$ effectors to contain HIV-1 in vivo. Inactivated whole virus-pulsed DC vaccines are promising means for treating people with chronic HIV-1 infection.

<p> Although the natural immune response to human immunodeficiency virus type 1 (HIV-1) is not effective for eradicating the virus, vigorous HIV-1-specific $CD4^+$ T-helper type 1 (Th1)-cell responses were shown to be associated with control of viremia and long-term non-progression in infected individuals [4-9]. Such a sustained HIV-1-specific cellular immunity might be a rational target for immunotherapy in patients with progressive infection. Early intervention with highly active antiretroviral therapy (HAART) during or shortly after acute infection is associated with enhanced HIV-1-specific $CD4^+$ Th1-cell responses [10,11]. In contrast, at a later stage, HAART leads to the decline of HIV-1-specific $CD4^+$ Th1-cell and $CD8^+$ cytotoxic T lymphocytes (CTL) responses [5,12,13], suggesting that the functional capacities of HIV-1-capturing antigen presentation cells (APCs) (which are required for the induction of the immune response) may be progressively lost along the course of the infection. Dendritic cells (DCs), the most potent APCs, play a pivotal role in the initiation and maintenance of immune responses against viruses [14] and have been found to be impaired in individuals with progressive HIV-1 infection [15-18]. Recent studies have demonstrated that the adoptive transfer of autologous DCs loaded in vitro with aldrithiol-2 (AT-2)-inactivated HIV-1 induced protective antiviral immunity in hu-PBL-SCID mice [2,3]. We had previously shown that a therapeutic vaccine made of AT-2-inactivated simian immunodeficiency virus (SIV) strain mac251 (SIVmac251)-loaded DCs led, in the absence of any antiviral therapy, to dramatic viral suppression in rhesus monkeys which were immunized 2 months after having been infected with SIVmac251 [1].

<p> Here we explored the toxicity and the efficacy of an AT-2-inactivated patient-matched HIV-1-loaded DC vaccine in patients with chronic HIV-1 infection.

<p> Seven weeks before the first vaccination (day 0), 20 chronically HIV-1-infected patients were enrolled in this study (Table 1). Two of them were excluded after inclusion: one (patient 9#) because his baseline plasma viral load was less than 5000 copies/ml by repeated viral-load measurements; the other one (patient 22#) because at the day 0 she had a blood platelet count below 100.000 cells/µl. Results were therefore based on 18 participants. Each of them was immunized subcutaneously at the root of both arms and both thighs with a total dose of 3×10^7 AT-2-inactivated HIV-1-loaded DCs. Two other injections with the same dose of thawed AT-2-inactivated HIV-1-

loaded DCs were administered at 2-week intervals. All patients completed the three doses of the DC vaccine and were followed up for one year without antiviral therapy.

<p> Among the 20 enrolled participants, 18 had completed three doses of the DC vaccines and were then followed up for one year. Therefore, results were based on 18 participants.

<p> The only clinical manifestation associated with the vaccine was an increase in the size of peripheral lymph nodes. The mean diameter (± SE) of left and right axillary and inguinal lymph nodes increased from 0.33 ± 0.11 cm before the first immunization to 1.17 ± 0.20 cm ($P < 0.01$) (second immunization), and to 1.61 ± 0.14 cm ($P < 0.001$) at week 4 (third immunization). The lymph node size remained significantly increased thereafter: 1.50 ± 0.19 cm at week 16 ($P < 0.001$), 1.67 ± 0.16 cm at week 32 ($P < 0.001$), and 1.06 ± 0.17 cm ($P < 0.01$) at week 52. No local or systemic side effect developed and no clinical AIDS or milder immunodeficiency-related symptoms (such as weight loss, unexplained fever, chronic diarrhea or oral candidiasis) occurred during the study period.

<p> Blood $CD4^+$ T-cell counts increased significantly as from week 4 but returned progressively to baseline level after week 16, whereas $CD8^+$ T-cell counts remained unchanged throughout the study (Fig. 1a). On the other hand, blood HIV-1 cellular viral load (CVL) and plasma viral load (PVL) levels decreased by 40% ($P < 0.001$) and 85% ($P < 0.001$) respectively over the 16 weeks following the first immunization. They remained thereafter stable until the end of the study (Fig. 1b). When looking at individual one-year changes of PVL, we observed that 8 patients had a PVL decline > 90% (thereafter referred to as strong responders), whereas the remaining 10 had a PVL decrease < 90% or a re-increase (referred to as weak responders) (Fig. 1c). Pre-vaccination levels and evolution profiles of $CD4^+$ or $CD8^+$ T-cell counts were not statistically different in strong responders (SR) and in weak responders (WR) (Fig. 1d). Baseline CVL or PVL were also not statistically different between SR and WR. PVL of SR decreased by 95% over the first 16 weeks ($P < 0.05$) and remained stable thereafter, while PVL of WR decreased by 70% over the same 16 weeks ($P < 0.05$) and reincreased as from week 32 (figure 1e). In SR, CVL decreased by 70% within the first 16 weeks ($P < 0.05$) and remained stable thereafter, whereas in WR, CVL remained unchanged over the year of the study (Fig. 1f).

<p> After having shown the existence of two patterns of viral-load response, we performed experiments to determine whether immunologic factors might be correlated with these patterns. Neutralizing antibodies against patient-matched autologous HIV-1 isolates were undetectable in 16 participants before vaccination; among them three became detected at low titers (1/10), one SR at weeks 8 and 16 and 2 other SR at week 16 only. Last two patients (one SR and one WR) had low titers (1/10) from day 0 to week 52.

<p> By a highly sensitive flow-cytometry-based intracellular cytokine (ICC) assay, we observed that HIV-1-specific inteleukine-2 (IL-2)-expressing and interferon-γ (IFN-γ)-expressing $CD4^+$ T cells increased 3-4 fold over the first 16 weeks in SR ($P < 0.01$) but remained unchanged in WR (Fig. 2a-b). Baseline frequencies of HIV-1-specific IL-2-expressing $CD4^+$ T cells were significantly higher in SR than in WR ($P < 0.01$) while baseline values of HIV-1-specific IFN-γ-expressing $CD4^+$ T cells were indistinguishable between SR and WR. On the other hand, HIV-1-specific IFN-γ-expressing (memory) $CD8^+$ T cells, which were similar in SR and WR before immunization, increased in parallel in both subgroups until week 16. They remained significantly increased until one year in SR while they returned to baseline levels at one year in WR (Fig. 2c). One-year changes of PVL strongly correlated with frequencies of HIV-1-specific IL-2- ($r^2 = 0.455$, $P < 0.01$) (Fig. 2d) or IFN-γ-expressing $CD4^+$ T cells ($r^2 = 0.603$, $P < 0.001$) (Fig. 2e). On the other hand, the correlation with frequencies of IFN-γ-expressing $CD8^+$ T cells was weaker ($r^2 = 0.220$, $P = 0.05$) (Fig. 2f).

<p> Using the combination of antigen-specific HLA tetramer binding and four-color flow cytometry in the 10 HLA-A*0201-positive participants (4 SR and 6 WR), we demonstrated that HIV-1-gag-specific perforin-expressing (effector) $CD8^+$ T cells increased 3 fold over the first 16 weeks ($P < 0.05$) and remained thereafter increased up to one year in SR. In contrast, these $CD8^+$ effectors had only a transient 2-fold increase at week 16 ($P < 0.05$) in WR (Fig. 3a). By one year, a significant linear correlation was observed between the frequency of HIV-1-gag-specific perforin-expressing $CD8^+$ T cells and the one-year change of PVL ($r^2 = 0.430$, $P < 0.05$) (Fig. 3b) or the frequency of HIV-1 -specific IL-2-expressing $CD4^+$ T cells ($r^2 = 0.906$, $P < 0.001$) (Fig. 3c) or the frequency of HIV-specific IFN-γ-expressing $CD4^+$ T cells ($r^2 = 0.843$, $P < 0.001$) (Fig. 3d).

<p> This is the first demonstration in humans of protective HIV-1-specific T-cell responses induced by a therapeutic vaccine in the absence of significant neutralizing antibodies. The frequencies of HIV-1 specific IL-2- and IFN-γ-expressing T cells following inactivated virus-pulsed DC stimulation, as measured by the ex vivo ICC assay, were much higher than those observed with methods where stimulators were HIV-1 peptides or recombinant proteins [5-9]. High frequencies of HIV-1-specific IFN-γ-expressing $CD8^+$ T cells have been also reported by others using an APC-mediated stimulator [19]. The significant correlation between viral suppression and durable increase in frequencies of HIV-1-specific $CD4^+$ Th1 cells and $CD8^+$ effectors observed in our immunized patients (figures 2a-b, 2d-e, and 3a-b) favors the notion that a strong virus-specific $CD4^+$ Th1-cell response is required to enable virus-specific $CD8^+$ effectors to contain HIV-1 replication in vivo.

<p> Given that the only initial (day 0) predictive makers for WR and SR was the frequency of HIV-1-specific IL-2-expressing (central memory) $CD4^+$ Th1 cells which has been shown to decline progressively along the course of the infection[5,7], it is conceivable that an early intervention by therapeutic vaccines could increase the probability of SR. This notion is also supported by the observation that dramatic viral suppression was indeed achieved by the immunization of Chinese macaques with early chronic SIV infection [1]. Whether the lack of response of $CD4^+$ Th1 cells observed in WR can be overcome by increasing/repeating vaccine doses remain to be determined. In this perspective, it is noteworthy that several studies concerning the nature history of human hepatitis virus B or C infection have shown a correlation between high levels of virus-specific $CD4^+$ Th1 cells and viral clearance [20-24]. Vaccines boosting virus-specific $CD4^+$ Th1 cells are promising treatments for humans with progressive HIV-1 infection and other chronic viral diseases.

<methlttl> Methods

<methlhd> **Patients.** This clinical phase I/II trial was approved on November 5, 2001 by the National Ethical Committee of the Brazilian Ministry of Health. HIV-1-positive volunteers signed a written informed consent before being enrolled in this study. To be included in this study the following criteria should be met seven weeks the first immunization: age ≥ 18, absence of pregnancy, HIV-1-seropositivity known since ≥ 1, no clinical AIDS, absence of antiretroviral therapy since ≥ 6 months, hemoglobin ≥ 10 g/dl, platelet ≥ 100,000/µl, blood CD4 cells ≥ 300/µl, plasma viral RNA ≥ 10,000 copies/ml, absence of other chronic diseases.

<methlhd> **Vaccine.** Patient-matched HIV-1 isolates were first obtained from CD8-depleted peripheral blood mononuclear cells (PBMCs) by viral culture (with autologous PBMC only) and inactivated by AT-2 (Sigma, St Louis, Missouri) as described [25]. One week before the immunization, 1-3 x 10^{10} PBMCs were collected by 3-hour leukapheresis from each patient and then transferred to a bio-safety (P3) clean room (class D) for a standardized 7-day culture of DCs [1]. Briefly, freshly collected PBMCs were subjected to plastic adherence at a density of 10^6 cells/cm^2 in the presence of 0.5% of clinical use human serum albumin (LFB, Les Ulis, France). After 2-hour incubation at 37°C in 5% CO2, non-adherent cells were removed by rinsing with sterile PBS buffer. Adherent cells were then cultured for 5 days in a complete medium containing clinical-grade CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 2000 U/ml GM-CSF (Schering-Plouph, Brinny, Ireland) and 50 ng/ml clinical-grade IL-4 (CellGenix). At day 5, 6 x 10^7 DCs (the remaining DCs were frozen in liquid nitrogen) were exposed to AT-2-inactivated autologous virus (10^9 viral particles/ml) at 37°C for 2 h; cells were then cultured for additional 2 days in the complete medium supplemented with clinical-grade cytokines IL-1β (10 ng/ml) (CellGenix), IL-6 (100 ng/ml) (CellGenix), and TNF-α (50 ng/ml) (CellGenix). At day 7, quality control (QC)

of DCs was performed by flow cytometry and trypan-blue staining; 3 x 10^7 QC-approved viable DCs were resuspended in 1 ml of sterile 0.9% chloride sodium solution and were ready for injection. Each patient received 4 subcutaneous infections of 0.25 ml in close proximity to left and right axillary and inguinal lymph nodes. Two booster injections with the patient-matched inactivated virus-loaded DCs (processed from the remaining frozen DCs) were given at the same dose at weeks 2 and 4.

<meth1hd> **Viral load assays.** Plasma HIV-1 RNA was measured by the Monitor kit (Roche Diagnostics, Summerville, New Jersey). Cell-associated HIV-1 DNA or supernatant HIV-1 RNA was quantified as described elsewhere [25,26].

<meth1hd> **Neutralizing antibody assay.** Neutralization against infection of healthy-donor PBMCs by patient-matched autologous virus was performed in patients sera as described [1]. As controls, cells were exposed to the same infectious dose of autologous virus or to the same virus dose pretreated with the sera taken from 5 healthy donors. The sensitivity of the neutralization assay was a titer of 10. When a serum sample was negative for neutralization at a 10-fold dilution, a value of 1 was assigned.

<meth1hd> **Flow cytometry assays.** Intracellular IL-2- and IFN-γ-expressing $CD4^+$ T cells or IFN-γ-expressing $CD8^+$ T cells following ex vivo stimulation with patient-matched DCs pulsed with AT-2-inactivated autologous virus were detected by previously described ICC assay [5,7]. HIV-1-specific $CD8^+$ effector T cells were measured by a combination of HLA-A*0201 HIV gag (SLYNTVATL) tetramer-PE (Beckman Coulter, Villepinte, France) and monoclonal antibodies against perforin-FITC, CD8-PerCP-Cy5.5, and CD3-APC (BD Biosciences Europe, Erembodegem, Belgium).

<meth1hd> **Statistic analysis.** Impaired data between different groups of patients or paired data before and at different time points after immunization were compared by the Mann-Whitney or the Wilcoxon test, respectively.

<received> Style tag for received and accepted dates (omit if these are unknown).

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<ack> **Competing interests statement.** The authors declare that they have no competing financial interests.

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<LEGEND> **Figure 1** Immunologic and virologic evolution of the 18 immunized patients. **a**, $CD4^+$ (○) or $CD8^+$ (□) T-cell counts (mean ± s.e.m). **b**, Geometric mean (± s.e.m) of plasma HIV-1 RNA (▲) or PBMC HIV-1 DNA (△). **c**, One-year individual changes (%) in plasma viral loads (PVL) from baseline in weak responders (WR) (< 90% PVL decrease; n = 10) (red lines) or in strong responders (SR) (> 90% PVL decrease; n = 8) (green lines). **d**, $CD4^+$ and $CD8^+$ T-cell counts of WR (○ and ◇) or of SR (● and ◆). **e**, PVL of WR (△) or of SR (▲). **f**, Cellular viral loads (CVL) of WR (○) or of SR (●). *P value (as compared to baseline levels) < 0.05; **P value < 0.01.

<LEGEND> **Figure 2** HIV-1-specfic T-cell immunity in the 18 immunized patients. **a**, HIV-1-specific interleukine-2 (IL-2)-expressing $CD4^+$ T cells of WR (△) or of SR (▲). **b**, HIV-1-specific interferon-γ (IFN-γ)-expressing $CD4^+$ T cells of WR (△) or of SR (▲). **c**, HIV-1-specific IFN-γ-expressing (memory) $CD8^+$ T cells of WR (▽) or of SR (▼). **d**, Correlation between HIV-1-specific IL-2-expressing $CD4^+$ T cells and changes of PVL from baseline. **e**, Correlation between HIV-1-specific IFN-γ-expressing $CD4^+$ T cells and changes of PVL from baseline. **f**, Correlation between HIV-1-specific IFN-γ-expressing $CD8^+$ T cells and changes of PVL from baseline. *P value (as compared to baseline levels) < 0.05.

<LEGEND> **Figure 3** HIV-1-gag specific perforin-expressing (effector) $CD8^+$ T cells in 10 immunized HLA A*0201$^+$ patients. **a**, HIV-1-gag-tetramer$^+$perforin$^+$ CD8 effectors (mean ± s.e.m) of WR (n = 6) (△) or of SR (n = 4) (□). **b**, Correlation between HIV-1-gag-tetramer$^+$perforin$^+$ CD8 effectors and changes of PVL from baseline. **c**, Correlation between HIV-1-gag-tetramer$^+$perforin$^+$ CD8 effectors and HIV-1-specific IL-2-expressing CD4 cells. **d**, Correlation between HIV-1-gag-tetramer$^+$perforin$^-$ CD8 effectors and HIV-1-specific IFN-□-expressing CD4 cells. *P value (as compared to baseline levels) < 0.05.

<TBLTTL> Table 1 **Clinical, immunologic, and virologic characteristics of study participants**

<TBLROW> Patient ID nb	Age	Sex	HLA A*0201	Months of known seropositivity	CD4 count (cells/μl)	Viral load (copies/ml)
<TBLROW> 2#	24	F	-	30	660	25,100
<TBLROW> 3#	24	F	+	42	490	33,100
<TBLROW> 4#	23	F	+	28	339	16,500
<TBLROW> 9#	30	M	-	37	225	2,249
<TBLROW> 10#	36	F	-	57	515	32,300
<TBLROW> 11#	25	F	-	25	522	46,800
<TBLROW> 13#	18	F	+	13	412	207,000
<TBLROW> 14#	26	F	-	50	455	22,000
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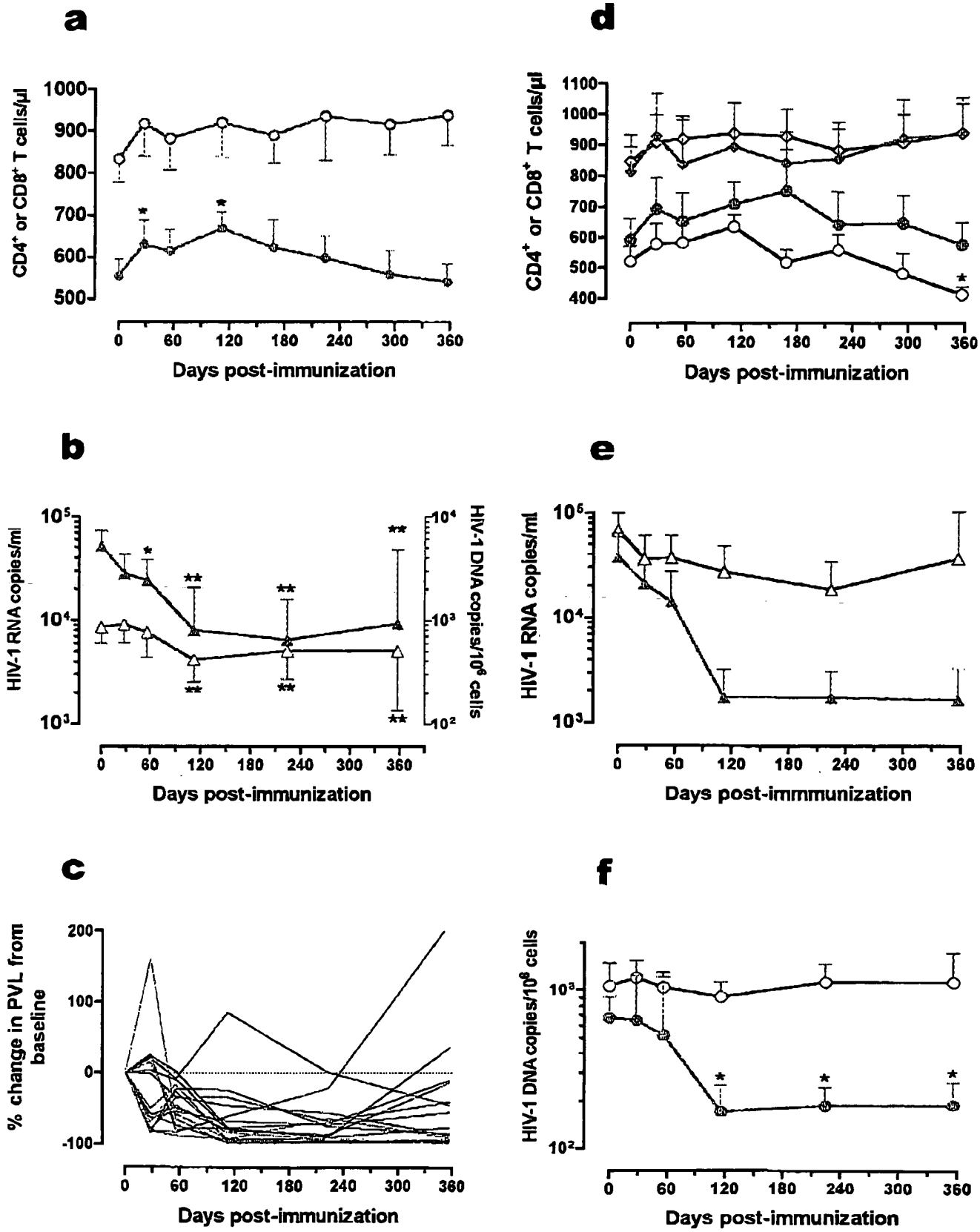
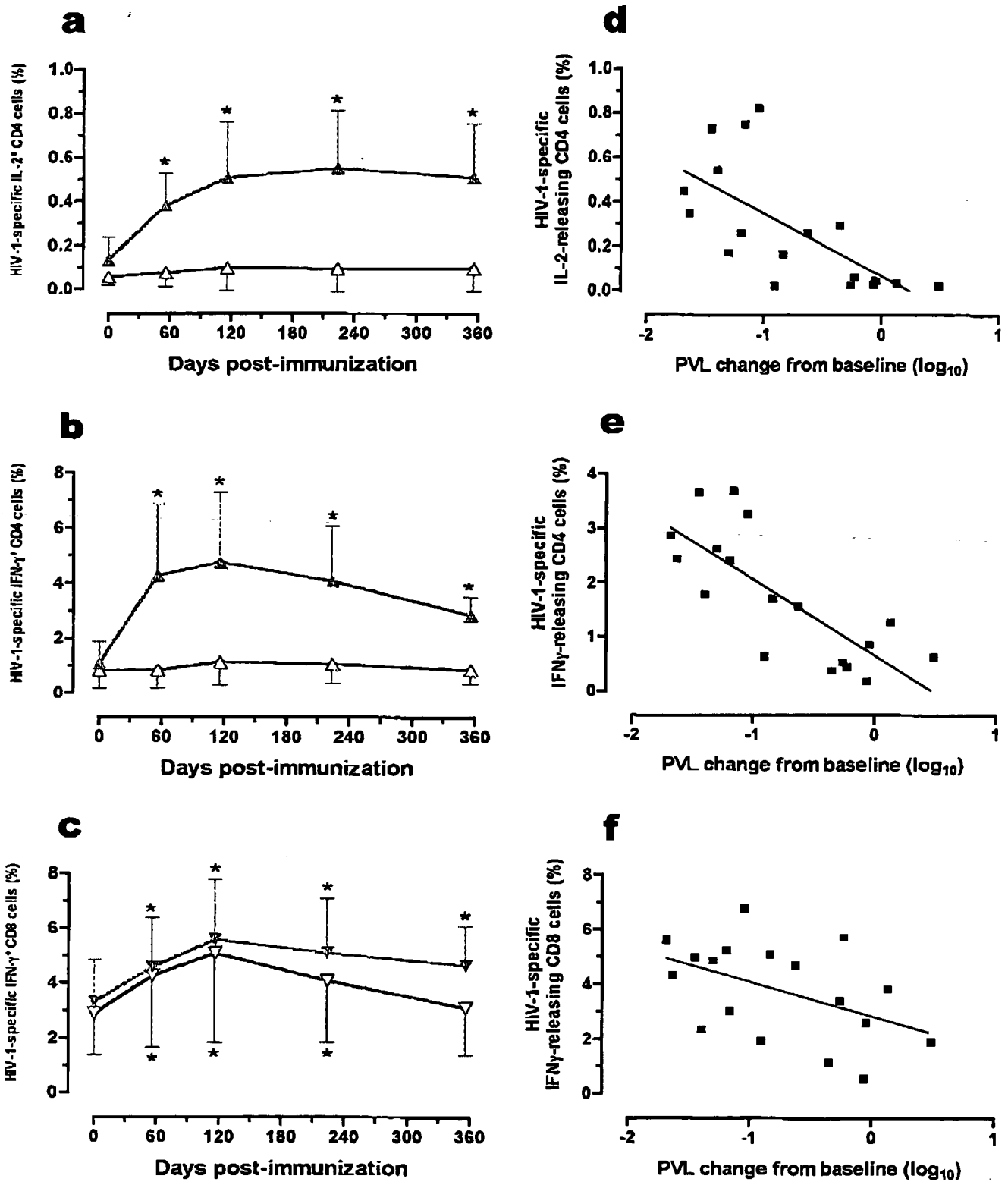
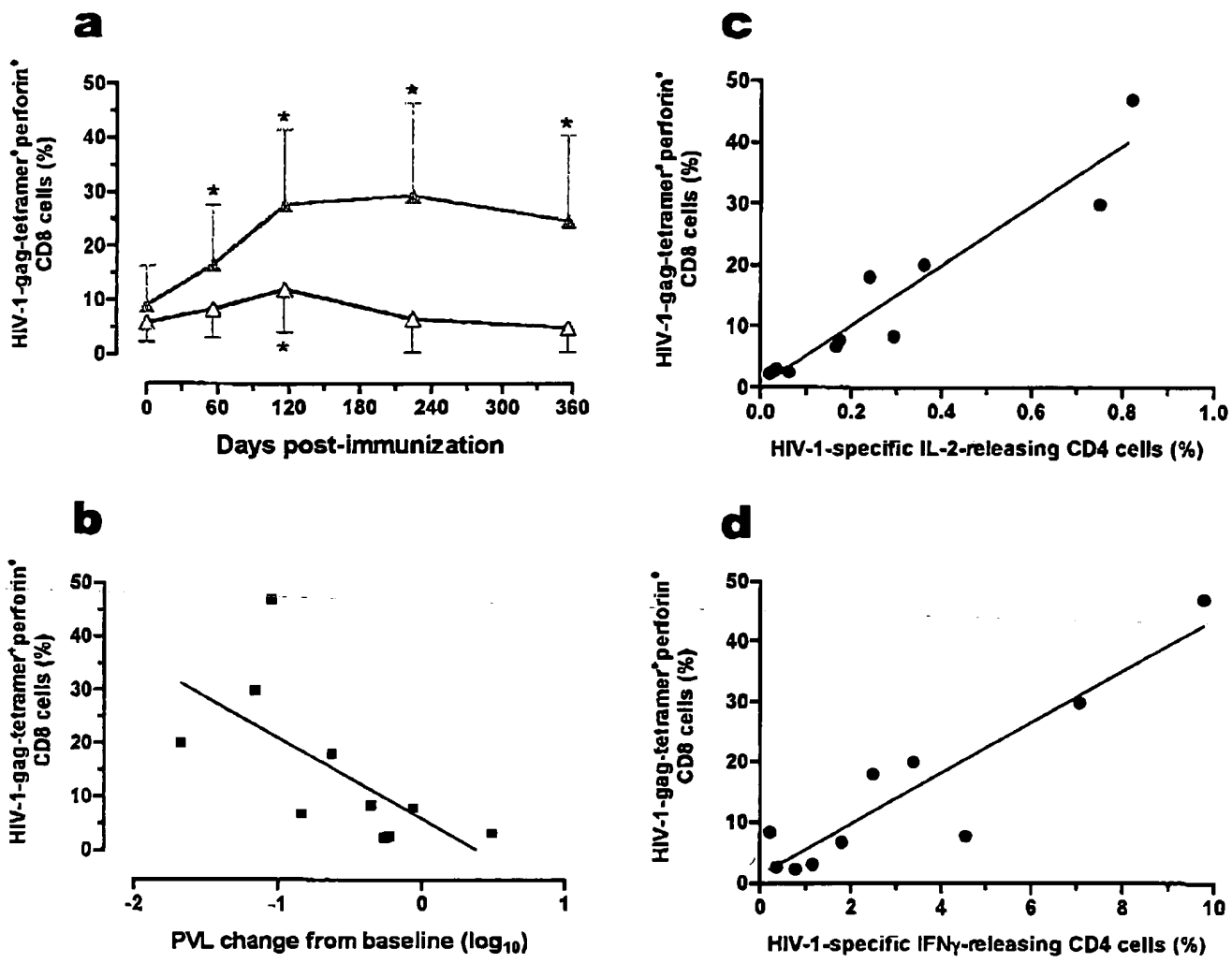
<TBLROW> 17#	27	F	+	38	1009	149,000
<TBLROW> 18#	24	F	-	17	722	11,100
<TBLROW> 19#	30	F	+	36	406	175,000
<TBLROW> 21#	21	F	-	13	270	95,500
<TBLROW> 22#	27	F	-	36	483	242,000
<TBLROW> 24#	41	M	+	23	476	20,900
<TBLROW> 27#	24	F	-	22	565	29,400
<TBLROW> 29#	25	F	+	41	528	41,600
<TBLROW> 31#	29	F	+	65	492	211,000
<TBLROW> 32#	29	F	+	18	721	13,200
<TBLROW> 33#	27	M	+	17	746	300,000


a
CD4+ or CD8+ T cells/µl
1000
900
800
700
600
500
0 60 120 180 240 300 360
Days post-immunization
d
CD4+ or CD8+ T cells/µl
1100
1000
900
800
700
600
500
400
0 60 120 180 240 300 360
Days post-immunization
b
HIV-1 RNA copies/ml
10⁵
10⁴
10³
HIV-1 DNA copies/10⁶ cells
10⁴
10³
10²
0 60 120 180 240 300 360
Days post-immunization
e
HIV-1 RNA copies/ml
10⁶
10⁵
10⁴
10³
0 60 120 180 240 300 360
Days post-immmunization
c
% change in PVL from baseline
200
100
0
-100
0 60 120 180 240 300 360
Days post-immunization
f
HIV-1 DNA copies/10⁶ cells
10³
10²
0 60 120 180 240 300 360
Days post-immunization


a
HIV-1-specific IL-2+ CD4 cells (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
60
120
180
240
300
360
Days post-immunization
d
HIV-1-specific IL-2-releasing CD4 cells (%)
-2
-1
0
1
PVL change from baseline (log10)
b
HIV-1-specific IFN-γ+ CD4 cells (%)
8
6
4
2
0
Days post-immunization
e
HIV-1-specific IFNγ-releasing CD4 cells (%)
3
PVL change from baseline (log10)
c
HIV-1-specific IFN-γ+ CD8 cells (%)
Days post-immunization
f
HIV-1-specific IFNγ-releasing CD8 cells (%)
PVL change from baseline (log10)


a
HIV-1-gag-tetramer⁺perforin⁺ CD8 cells (%)
Days post-immunization
b
HIV-1-gag-tetramer⁺perforin⁺ CD8 cells (%)
PVL change from baseline (log10)
c
HIV-1-gag-tetramer⁺perforin⁺ CD8 cells (%)
HIV-1-specific IL-2-releasing CD4 cells (%)
d
HIV-1-gag-tetramer⁺perforin⁺ CD8 cells (%)
HIV-1-specific IFNγ-releasing CD4 cells (%)

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In Vitro Human Immunodeficiency Virus Eradication by Autologous CD8$^+$ T Cells Expanded with Inactivated-Virus-Pulsed Dendritic Cells

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Despite significant immune recovery with potent highly active antiretroviral therapy (HAART), eradication of human immunodeficiency virus (HIV) from the bodies of infected individuals represents a challenge. We hypothesized that an inadequate or inappropriate signal in virus-specific antigen presentation might contribute to the persistent failure to mount efficient anti-HIV immunity in most HIV-infected individuals. Here, we conducted an in vitro study with untreated (n = 10) and HAART-treated (n = 20) HIV type 1 (HIV-1) patients which showed that pulsing of monocyte-derived dendritic cells (DC) with aldrithiol-2-inactivated autologous virus resulted in the expansion of virus-specific CD8$^+$ T cells which were capable of killing HIV-1-infected cells and eradicating the virus from cultured patient peripheral blood mononuclear cells independently of the disease stages and HAART response statuses of the patients. This in vitro anti-HIV effect was further enhanced by the HIV protease inhibitor indinavir (at a nonantiviral concentration), which has been shown previously to be able to up-regulate directly patient T-cell proliferation following immune stimulation. However, following a 2-day treatment with culture supernatant derived from immune-activated T cells (which mimics an in vivo environment of HIV-disseminated and immune-activated lymphoid tissues), DC lost their capacity to present de novo inactivated-virus-derived antigens. These findings provide important information for understanding the establishment of chronic HIV infection and indicate a perspective for clinical use of DC-based therapeutic vaccines against HIV.

Although the introduction of highly active antiretroviral therapy (HAART) including at least one human immunodeficiency virus (HIV) protease inhibitor (PI) allows dramatic decreases in plasma HIV RNA loads and significant recovery of the T-cell compartment in the majority of patients (2, 5), HIV eradication by prolonged HAART treatment appears to be unlikely due to the persistence of a cellular reservoir of infectious HIV (4, 7, 13). On the other hand, HAART-treated patients fail to mount anti-HIV immunity, as evidenced by the rapid viral rebound observed in almost all patients after discontinuation of HAART (6, 10, 11) or by a maintained high viral load in patients experiencing a virologic failure despite significant T-cell recovery (8, 15–19). We hypothesized that the persistent failure in mounting anti-HIV immunity in untreated or HAART-treated patients might be caused by an inadequate or inappropriate signal in virus-specific antigen presentation, possibly resulting from a disturbance in the generation and/or function of antigen-presenting cells (APCs) in chronically immune-activated lymphoid organs or tissues of HIV-infected patients.

We report here that monocyte-derived dendritic cells (DC) (the most potent APCs capable of priming major histocompatibility complex class I- and II-restricted antigen-specific T-cell responses) pulsed with inactivated autologous virus can result in the expansion of virus-specific CD8$^+$ T cells capable of killing HIV-infected cells and suppressing HIV type 1 (HIV-1) replication. Since we previously observed that HIV PIs even at nonantiviral doses could restore proliferative responses of patient T cells (19), we hypothesized that PIs might be helpful in enhancing the generation of virus-specific effector cells. Indeed, a combination of inactivated-virus-pulsed DC and the HIV PI indinavir (at a nonantiviral concentration) resulted in an ample expansion of virus-specific CD8$^+$ T cells which was sufficient to eradicate HIV-1 in peripheral blood mononuclear cells (PBMC) taken from HIV-infected patients.

MATERIALS AND METHODS

Study populations. A total of 30 HIV-1-infected adults were selected from our outpatient clinic. Ten of them had never received antiretroviral drugs, had a CD4 cell count of between 200 and 600 cells/μl, and had a plasma viral load of between 4 and 6 $\log_{10}$ HIV RNA equivalent (eq) copies/ml (Quantiplex HIV-1 3.0; Bayer, Emeryville, Calif.). Ten other patients had received prolonged HAART (>3 years), had a CD4 cell count of between 300 and 700 cells/μl, and had a sustained undetectable plasma HIV-1 RNA level (<50 eq copies/ml; Quantiplex HIV-1 3.0) for at least 30 months. The last 10 patients had received prolonged HAART and had a CD4 cell count of between 300 and 700 cells/μl but had maintained a plasma viral load of between 4 and 6 $\log_{10}$ HIV RNA eq copies/ml (Table 1). Informed consent was obtained from all participants.

DC generation. PBMC were isolated from fresh whole citrate-treated blood obtained by venipuncture from HIV-1-infected patients using Ficoll-Hypaque (Eurobio, Les Ulis, France) density gradient centrifugation. After four washes with Hanks' balanced salt solution (Hanks' buffer), monocytes were enriched by negative immunoselection using a commercial kit (Dynal, Great Neck, N.Y.). Isolated monocytes which were 90% pure (CD14$^+$ cells) were plated at 10^6/ml of serum-free AIM-V medium (Life Technologies, Grand Island, N.Y.) in flasks (Nunc, Roskilde, Denmark). Cells were then cultured for 5 days in AIM-V medium supplemented with 250 ng of granulocyte-macrophage colony-stimulat-

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TABLE 1. Characteristics of 30 HIV-1-infected adults (10 who were naive for antiviral treatment, 10 HAART-treated virologic responders, and 10 HAART-treated virologic nonresponders)

Patient no.	Sex[a]	Antiretroviral regimen[b]		Mo of HAART	CD4 cell count (cells/μl)	Plasma viral load (log_{10} eq copies/ml)
		Initial	Modification (mo[c])			
1	M	Naive			247	4.85
2	M	Naive			262	4.62
3	F	Naive			302	4.24
4	M	Naive			374	5.14
5	M	Naive			395	4.88
6	M	Naive			415	4.21
7	F	Naive			421	4.38
8	M	Naive			467	5.22
9	F	Naive			541	4.39
10	M	Naive			557	5.01
11	M	3TC-d4T-IDV	None	40	332	<1.70
12	M	3TC-d4T-IDV	None	42	426	<1.70
13	F	3TC-d4T-IDV	None	40	441	<1.70
14	M	3TC-d4T-IDV	None	38	449	<1.70
15	M	3TC-d4T-IDV	None	41	483	<1.70
16	F	3TC-d4T-IDV	3TC-ZDV-IDV (1)	38	559	<1.70
17	M	3TC-d4T-IDV	3TC-ZDV-IDV (5)	43	566	<1.70
18	F	3TC-d4T-IDV	None	38	605	<1.70
19	M	3TC-d4T-IDV	None	40	612	<1.70
20	M	3TC-d4T-IDV	None	40	696	<1.70
21	M	3TC-d4T-IDV	None	39	311	5.44
22	M	3TC-d4T-IDV	3TC-ZDV-IDV (4)	41	385	4.26
23	M	3TC-d4T-IDV	3TC-d4T-RTV-SQV (6)	43	397	4.79
24	M	3TC-d4T-IDV	None	45	401	4.28
25	M	3TC-d4T-IDV	3TC-d4T-RTV-SQV (8)	42	409	5.87
26	M	3TC-d4T-IDV	3TC-d4T-RTV-SQV (8)	42	523	4.67
27	M	3TC-d4T-IDV	None	41	569	5.63
28	F	3TC-d4T-IDV	3TC-d4T-RTV-SQV (7)	44	646	5.18
29	M	3TC-d4T-IDV	3TC-ZDV-IDV (5)	42	654	4.47
30	M	3TC-d4T-IDV	3TC-ZDV-IDV (4)	41	672	5.15

[a] M, male; F, female.
[b] ZDV, zidovudine; 3TC, lamivudine; d4T, stavudine; IDV, indinavir; RTV, ritonavir; SQV, saquinavir.
[c] Indicates the time after which the initial antiretroviral regimen was modified because of side effects of IDV or d4T.

ing factor (GM-CSF) (R&D Systems, Minneapolis, Minn.) per ml and 100 ng of interleukin-4 (IL-4) (R&D Systems) per ml. Following this culture period, nonadherent cells were determined to be >90% immature DC based on their morphology and their expression of CD1a, CD11c, CD40, CD80, CD86, CD4, and HLA-DR assessed by direct immunofluorescence flow cytometry (see below).

Induction of virus-specific T-cell response. Viruses were isolated by coculture of phytohemagglutinin (Sigma, St. Louis, Mo.)-stimulated HIV-negative donor PBMC with patient $CD4^+$ T cells (3). Viral isolates were inactivated with 250 μM aldrithiol-2 (AT-2) (Sigma) for 1 h at 37°C in order to preserve the intact native conformation and fusogenic activity of HIV Env protein gp120 (24). Immature DC were pulsed with AT-2-inactivated autologous isolate (50 ng of p24) for 2 h at 37°C. After three washes with Hanks' buffer, virus-pulsed DC were cultured in AIM-V medium supplemented with 100 ng of GM-CSF per ml, 5 ng of IL-4 per ml, 50 ng of tumor necrosis factor alpha (TNF-α) (R&D Systems) per ml, and 1,000 U of alpha interferon 2b (IFN-α-2b) (Schering-Plough, Brinny, Ireland) per ml for 3 days. In certain experiments, immature DC were treated for 2 h before or after pulsing with inactivated autologous virus with the culture supernatant collected from anti-CD3/CD28 antibody-stimulated normal donor T cells. Peripheral blood lymphocytes (PBL) were freshly prepared from nonadherent PBMC. PBL (3×10^6) were stimulated on day 0 and restimulated on day 7 with virus-pulsed autologous DC (at a stimulator/responder ratio of 1:3) in the absence or the presence of a nonantiviral concentration of PI (10 nM indinavir; Merck Research Laboratories, West Point, Pa.). PBL (10^6/ml) were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Eurobio), nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (referred as complete medium). Exogenous IL-2 (25 IU/ml; Roche Molecular Biomedicals, Mannheim, Germany) was added every 3 to 4 days.

Proliferation assay. At day 14 of culture, 10^5 PBL were added to AT-2-inactivated virus-pulsed DC at stimulator/responder ratios of 1:3, 1:10, 1:30, and 1:100 in quadruplicate wells in 96-well round-bottomed microtiter plates (Nunc). After 24 h, cells were pulsed with 0.037 MBq (1 μCi) of [*methyl*-^{3}H]thymidine (Amersham Pharmacia Biotech, Aylesbury, United Kingdom) per well, and incubation was continued for an additional 18 h. Cells were harvested on glass fiber filters by an automated multisample harvester; filters were then put in tubes with liquid scintillation fluid, and radioactivity was counted on a beta-scintillation counter. Net counts per minute were calculated by subtracting the counts per minute of control PBL which were cultured with unloaded autologous DC for 14 days and then exposed to unloaded autologous DC for an additional 24 h prior to addition of [*methyl*-^{3}H]thymidine.

Cytotoxicity assay. Autologous Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were infected with recombinant vaccinia virus containing a *gag* gene of HIV-1 (25). Vaccinia virus-infected B-LCL were incubated with 100 μCi of ^{51}Cr in a total volume of 200 μl for 2 h at 37°C before use as targets. The effector cells were derived from HIV-infected patient PBL cultured with inactivated-virus-pulsed autologous DC for 14 days as described above. Targets were washed and seeded at 5×10^3 cells/well at an effector/target ratio of 10:1 in quadruplicate and assayed for cytotoxicity in a standard chromium release assay. The percentage of specific lysis was calculated by subtracting the specific ^{51}Cr release of wild-type vaccinia virus-infected targets (controls) (25). Anti-CD4 and anti-CD8 monoclonal antibodies (clones RPA-T4 and RPA-T8; PharMingen) were used for blocking analysis.

Anti-HIV activity assay. The direct anti-HIV activity of PBL expanded with virus-pulsed DC with or without PI was evaluated with autologous patient PBMC using our recently described assay system (26) with slight modifications. Briefly, patient PBMC were superinfected with 100 50% tissue culture infective doses of autologous viral isolate. After three washes with Hanks' buffer, cells were stimulated with 0.5 μg of anti-CD3 monoclonal antibodies (Becton Dickinson France, Le Pont-de-Claix, France) per ml plus 100 ng of anti-CD28 monoclonal antibodies (PharMingen, Los Angeles, Calif.) per ml for 24 h. After additional washes, PBL expanded with virus-pulsed DC with or without PI were added to

superinfected patient PBMC at an effector/target ratio of 1:1 (the lowest ratio for demonstrating a significant antiviral activity in most patient T cells). The coculture was maintained in complete medium supplemented with 20 IU of IL-2 per ml. Half of the culture supernatant was replaced with fresh medium every 2 to 3 days. At day 15, cells were collected for measuring the proviral HIV DNA load and supernatants were harvested for measuring the cell-free HIV RNA concentration. Anti-CD4 and anti-CD8 monoclonal antibodies (PharMingen) were used for blocking analysis.

Viral quantitation assay. Culture supernatant viral concentrations were determined by measuring cell-free HIV RNA by multiple-primer-induced overlapping amplification assay with a detection threshold of 10 eq copies/ml (21). Proviral DNA was determined by a quantitative PCR assay (22) with several recent modifications. Briefly, 2×10^6 cells were used for cellular DNA purification with a commercial kit (QIAamp; Qiagen, Hilden, Germany). Purified DNA equivalent to that of 10^6 cells was used for HIV-specific amplification as described previously (22). Four standard dilutions (10, 100, 1,000, and 10,000 copies in HIV-negative donor PBMC DNA equivalent to that of 10^6 cells) of HIV-1 DNA plasmid (pBH10-R3) were amplified in parallel as external standards. At the same time, each standard and sample DNA (amount equivalent to that of 10^4 cells) was amplified in parallel as calibrators (DNA input control) using the primer pair 5'-GCCGGGAAATCGTGCGTTGACATT-3' (sense; nucleotides [nt] 2280 to 2301 of the β-actin genomic sequence HUMACCYBB; GenBank accession number M10277) and 5'-GATGGAGTTGAAGGTAGTTTCG TG-3' (antisense; nt 2606 to 2583 of HUMACCYBB). After amplification, 10 μl of HIV or β-actin reaction product of each sample was distributed into streptavidin-coated 96-well microtiter plates (Roche Molecular Biochemicals) preincubated with biotin-labeled probe specific for HIV-1 (21) or β-actin (5'-TGTGC TGTGGAAGCTAAGTCCTGCCCTCATTT-5'; nt 2522 to 2553 of HUMAC CYBB). Quantitation was performed by a hybridization-linked enzyme-linked immunosorbent assay as previously described (21). The sensitivity of the assay reached 5 HIV copies per 10^6 cells, with a quantitative range of 5 to 10^5 HIV DNA copies per 10^6 cells and intra- and interassay variabilities of less than 0.05 and 0.08 $\log_{10}$ copies, respectively.

Flow cytometry. Counts of $CD4^+$ and $CD8^+$ T cells ($CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$), monocytes ($CD14^+$), and DC ($CD1a^+$, $CD11c^+$, $CD40^+$, $CD80^+$, $CD83^+$, $CD86^+$, $CD4^+$, and HLA-DR^+) were assessed by flow cytometry analysis (FACScan; Becton Dickinson, San Jose, Calif.) using a panel of direct fluorescence-labeled monoclonal antibodies: CD3peridinin chlorophyll protein (PerCP), CD4-fluorescein isothiocyanate (CD4-FITC), CD8-phycoerythrin (CD8-PE), and CD14-PE (Becton Dickinson) and CD1a-FITC, CD11c-PE, CD40-FITC, CD80-PE, CD83-FITC, CD86-FITC, and HLA-DR–PE (PharMingen).

Statistical analysis. Unpaired data for different groups of patients or paired data for different in vitro treatments were compared by the Mann-Whitney test or the Wilcoxon test, respectively.

RESULTS

Proliferation of patient T cells following stimulation with virus-pulsed autologous DC. Viruses were isolated from 10 untreated asymptomatic HIV-seropositive patients (CD4 cell count, 200 to 600 cells/μl; plasma HIV RNA load, 4 to 6 $\log_{10}$ eq copies/ml) and 20 patients treated by prolonged HAART (>3 years) (CD4 cell count, 300 to 700 cells/μl; 10 patients with virologic response [plasma HIV RNA load, <50 $\log_{10}$ eq copies/ml] and 10 patients with virologic resistance [plasma HIV RNA load, 4 to 6 $\log_{10}$ eq copies/ml]) (Table 1). To mimic the antigen capture by DC in peripheral tissues, immature DC (i.e., competent in antigen capture) generated by culturing patient blood monocytes with GM-CSF and IL-4 for 5 days were pulsed with autologous viral isolates inactivated by AT-2, which preserves the intact native conformation and fusogenic activity of HIV Env protein (24). To model the presentation of antigens in lymphoid tissue, virus-pulsed DC were matured in the presence of TNF-α and IFN-α for an additional 3 days to maximize their T-cell-stimulatory activity (27, 29). Matured virus-pulsed DC were then used to stimulate autologous PBL at a stimulator/responder ratio of 1:3 in the absence or presence of PI at a nonantiviral concentration (10 nM indinavir). By day 7 of coculture, PBL were restimulated with the same virus-pulsed DC for an additional 7 days. At day 14, proliferation was measured by incubating PBL with virus-pulsed DC at stimulator/responder ratios of 1:3 to 1:100.

Inactivated virus-pulsed DC stimulated [*methyl*-^{3}H]thymidine incorporation by autologous PBL from both untreated and HAART-treated patients independently of their blood CD4 cell counts and plasma viral loads ($P > 0.5$), while this DC-mediated T-cell proliferation was significantly enhanced by the presence of PI (at a nonantiviral dose) ($P < 0.001$) (Fig. 1A). Phenotype analysis by flow cytometry showed that both $CD4^+$ and $CD8^+$ T cells were equally stimulated by virus-pulsed DC in the presence or absence of PI (Fig. 1B).

HIV-1 *gag*-specific CTL activity following stimulation with virus-pulsed autologous DC. We next evaluated HIV-specific cytotoxic-T-lymphocyte (CTL) activity of autologous PBL expanded with inactivated-virus-pulsed DC using as target cells autologous B-LCL infected by recombinant vaccinia virus containing a *gag* gene of HIV-1 as described previously (25). HIV *gag*-specific B-LCL killing was up-regulated by autologous PBL stimulated with virus-pulsed DC (at an effector/target ratio of 10:1) in both untreated and HAART-treated patients independently of their blood CD4 cell counts and plasma viral loads ($P > 0.4$). Such a *gag*-specific CTL activity was significantly enhanced ($P < 0.01$) by the presence of indinavir (10 nM) (Fig. 2A). Similar enhancement by PI was also observed with PBL stimulated with virus-pulsed DC alone when the effector/target ratio was increased to 50:1 (data not shown). This CTL-mediated B-LCL killing was executed exclusively by $CD8^+$ T cells, since cell killing was blocked by the addition of anti-CD8 antibodies whereas it was unaffected by the addition of anti-CD4 antibodies (Fig. 2B).

Anti-HIV activity of patient T cells following stimulation with virus-pulsed autologous DC. Having observed that virus-pulsed DC were capable of stimulating the proliferation and CTL activity of autologous T cells from both untreated and HAART-treated patients regardless of their CD4 cell count and level of HIV viremia, we then examined the direct antiviral activity of autologous T cells expanded by virus-pulsed DC in the presence or the absence of PI. To minimize the variation in the frequency of PBMC harboring infectious HIV among untreated and HAART-treated patients, all patient PBMC were superinfected with the same dose (100 50% tissue culture infective doses) of autologous isolates as described previously (26). The total HIV proviral DNA concentrations (means ± standard deviations [SDs]) measured before and after 12 h of superinfection were 2.9 ± 0.5 (range, 2.1 to 3.8) and 4.1 ± 0.2 (range, 3.7 to 4.5) $\log_{10}$ copies per million PBMC, respectively. To mimic immune activation in lymphoid organs, superinfected patient PBMC were stimulated with anti-CD3 and anti-CD28 antibodies and then cocultured with autologous PBL expanded with virus-pulsed DC with or without PI at an effector/target ratio of 1:1. Unloaded DC-treated T cells were used in parallel as a control. Cell-associated proviral DNA and supernatant viral RNA concentrations were measured by previously described quantitative assays (21, 22). The proviral DNA load (copies per 10^6 cells) was decreased by 2 $\log_{10}$ ($P < 0.001$) in autologous T cells expanded with virus-pulsed DC without PI, whereas it was decreased by >3 $\log_{10}$ ($P < 0.001$)

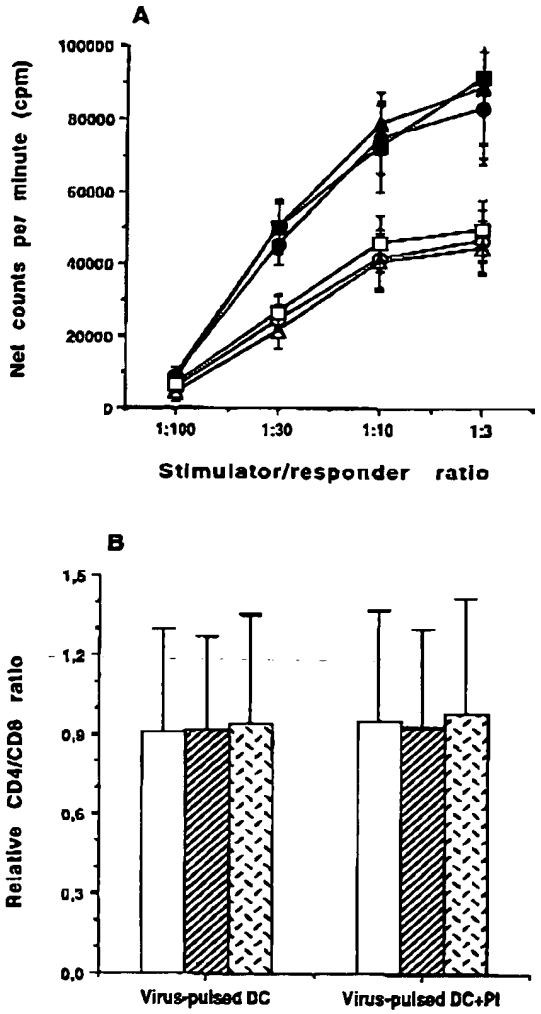

FIG. 1. Proliferation of patient T cells following stimulation with inactivated-virus-pulsed autologous DC in the absence or presence of HIV PI (indinavir, 10 nM). (A) Mean (± SD) [^{3}H]thymidine incorporation in T cells from untreated patients in the absence (□) or presence (■) of PI, in T cells from HAART-treated plasma viral load responders in the absence (△) or presence (▲) of PI, and in T cells from plasma viral load nonresponders in the absence (○) or presence (●) of PI. (B) Mean (± SD) relative CD4/CD8 ratio in T cells from untreated patients (□), plasma viral load responders (▨), and plasma viral load nonresponders (▩). The baseline CD4/CD8 ratio in the absence of stimulation was normalized to 1. The baseline ranges of the $CD3^+$ $CD4^+$ phenotype in untreated patients, plasma viral load responders, and plasma viral load nonresponders were 15 to 32%, 24 to 53%, and 21 to 41%, respectively.

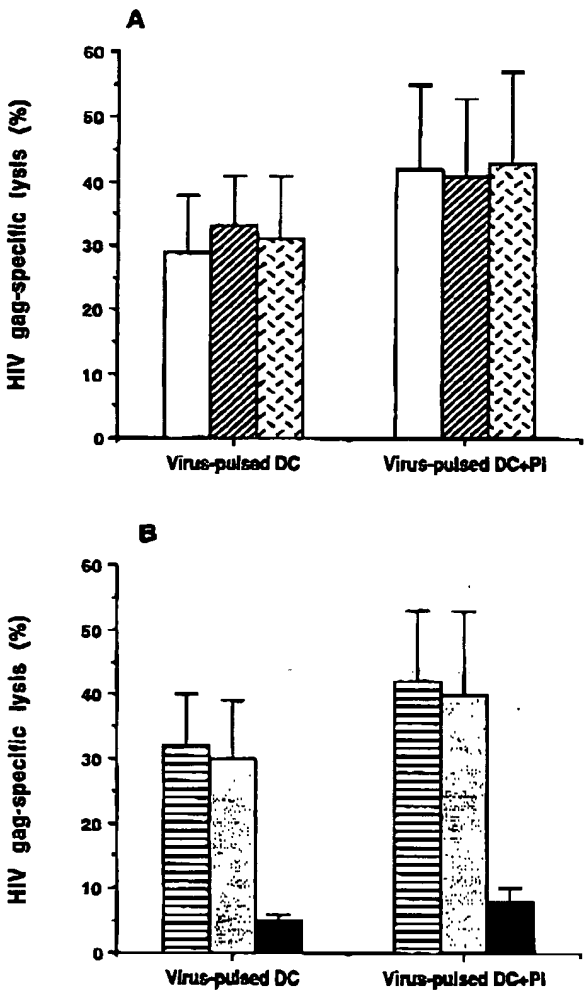

FIG. 2. HIV-1 *gag*-specific CTL activity in patient T cells expanded by inactivated-virus-pulsed autologous DC in the absence or presence of indinavir. (A) Mean (± SD) percent specific lysis (at an effector/target ratio of 10:1) of autologous B-LCL targets (infected with recombinant vaccinia virus containing a HIV-1 *gag* gene) by T cells stimulated with virus-pulsed DC with or without PI. T cells were from untreated patients (□), plasma viral load responders to HAART (▨), and plasma viral load nonresponders to HAART (▩). (B) Mean (± SD) percent specific lysis from all patients in the absence of antibodies (□) or in the presence of blocking antibodies against CD4 (▨) or CD8 (■). The background percent *gag*-specific lysis using unloaded DC-treated T cells was <10%.

(i.e., below the detection threshold of 5 copies/10^6 cells) in T cells expanded with virus-pulsed DC with PI. On the other hand, HIV RNA in the supernatants of the same cultures was decreased by 4 $\log_{10}$ ($P < 0.001$) and >6 $\log_{10}$ (i.e., below the detection threshold of 10 copies/ml) in these two situations (Fig. 3A and B). Optimum suppressions of proviral DNA and supernatant RNA to levels below the detection threshold were also obtained by patient T cells stimulated with virus-pulsed DC alone when the effector/target ratio was increased to 5:1 (data not shown). Addition of anti-CD8 antibodies abolished these antiviral activities, while addition of anti-CD4 antibodies

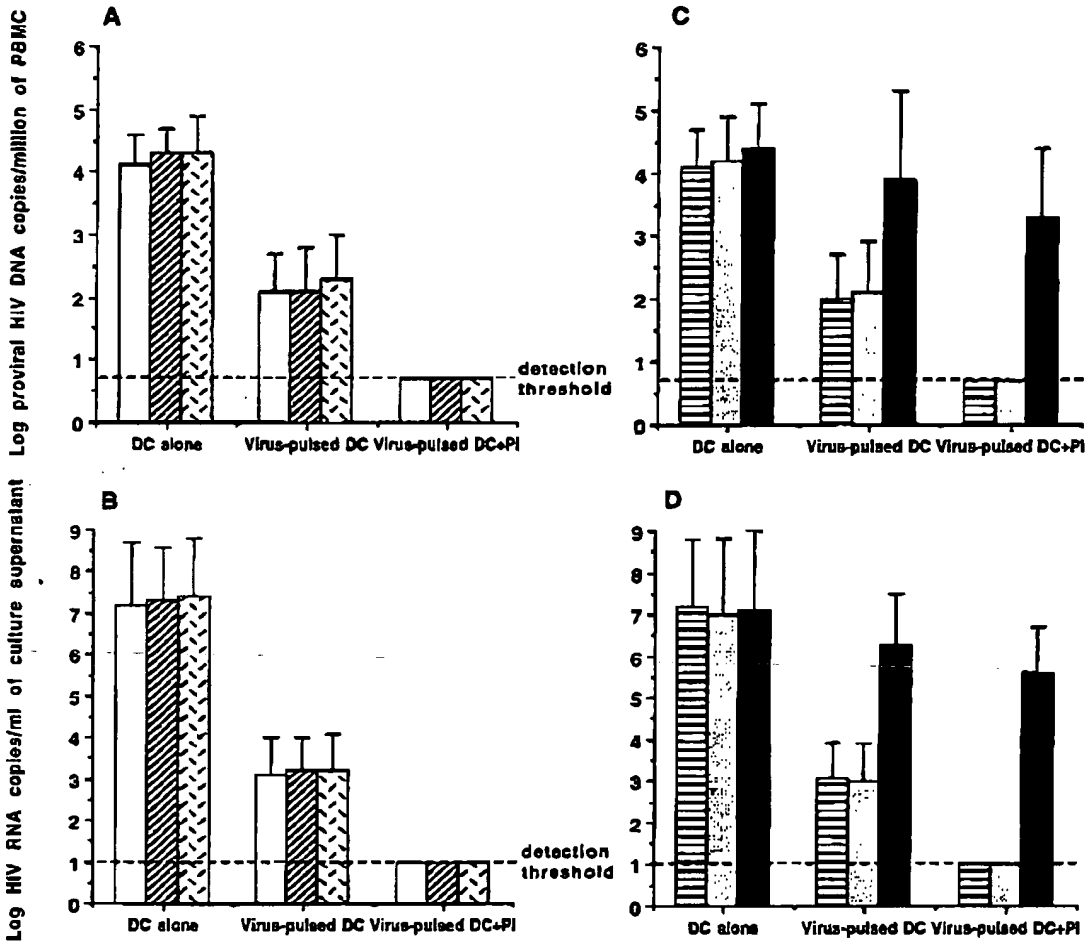

FIG. 3. Quantitative analysis of anti-HIV activity of patient T cells stimulated with inactivated-virus-pulsed autologous DC in the absence or presence of PI. Each result is the mean (± SD) number of proviral HIV DNA copies/10⁶ cells (A and C) or the mean (± SD) number of supernatant HIV RNA copies per milliliter (B and D) in the coculture of autologous virus-pulsed-DC-stimulated T cells and superinfected T cells from untreated patients (□), plasma viral load responders to HAART (▨), and plasma viral load nonresponders to HAART (▩) or from all patients in the absence of antibodies (⊟) or the presence of blocking antibodies against CD4 (▣) or CD8 (■).

did not have any effect on the clearance of proviral HIV DNA or supernatant HIV RNA (Fig. 3C and D). Again, the antiviral activity of autologous CD8+ T cells expanded by virus-pulsed DC or by virus-pulsed DC plus PI (indinavir, 10 nM) was achieved equally in untreated and HAART-treated patients whatever their CD4 cell counts and viral load levels ($P > 0.3$). The cultures showing undetectable proviral DNA and supernatant HIV-1 RNA were further cocultured with phytohemagglutinin-stimulated normal donor PBMC for 30 days. No infectious virus was recovered from any of these cultured patient T cells that had demonstrated undetectable proviral DNA and supernatant viral RNA.

DC functions following treatment with activated-T-cell-derived supernatant. Since the immune-activated lymphoid organs and tissues are the major sites for HIV replication and dissemination, we questioned whether DC could uptake and process HIV and/or present HIV antigens to effector T cells in such an immune-activated environment. Immature DC were pretreated for 2 days with the culture supernatant derived from T cells stimulated with anti-CD3/CD28 antibodies for 7 days, and then proliferation, CTL, and antiviral activities were analyzed as described above. When pretreated with activated-T-cell supernatant before the virus pulse, patient DC lost their capacity to stimulate proliferation, *gag*-specific CTL response, and HIV-expressing cell killing of autologous T cells. However, these DC functions were preserved when the activated-T-cell supernatant was added to DC after pulsing with inactivated virus (Fig. 4). Flow cytometric analysis showed a supermatu-

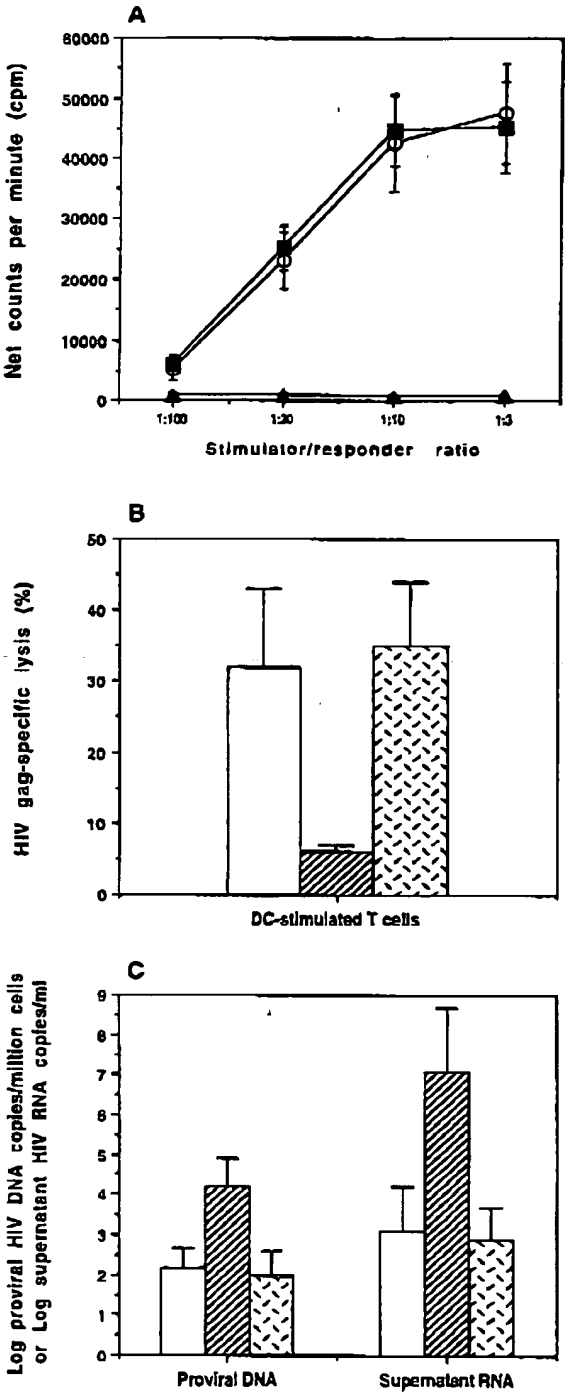

FIG. 4. Functions of DC following treatment with activated-T-cell supernatant. (A) Mean (± SD) [^{3}H]thymidine incorporation in patient T cells stimulated with virus-pulsed DC (○) or DC pretreated with activated-T-cell supernatant before (▲) or after (■) pulsing with inactivated autologous virus. (B) Mean (± SD) percent HIV *gag*-specific lysis (at an effector/target ratio of 10:1) of autologous B-LCL targets by patient T cells expanded with virus-pulsed DC (□) or DC pretreated with activated-T-cell supernatant before (▨) or after (▩) pulsing with inactivated autologous virus. (C) Mean (± SD) number of proviral HIV DNA copies/10^6 cells or supernatant HIV RNA copies per milliliter in the coculture of superinfected T cells with autologous T cells expanded with virus-pulsed DC (□) or DC pretreated with activated-T-cell supernatant before (▨) or after (▩) pulsing with inactivated autologous virus.

ration phenotype (up-regulated expression of CD40, CD80, CD83, CD86, and major histocompatibility complex class II) of DC following exposure to activated-T-cell supernatant.

DISCUSSION

Our data provide the first evidence that a high frequency of PBMC harboring HIV can be eradicated in vitro by cultured patient T cells expanded with inactivated-virus-pulsed autologous DC. This potent antiviral activity of patient T cells stimulated with virus-pulsed DC is CD8 dependent and independent of the patient's disease stage and treatment status. Treatment of patient DC with activated-T-cell supernatant results in the loss of their integrated APC functions to present de novo viral antigens. These findings indicate that a disturbance in the presentation of viral antigens is most likely the cause of failure in mounting an efficient anti-HIV immunity in untreated HIV-seropositive individuals as well as in HAART-treated patients despite a significant improvement of T-cell reactivity (9, 14). The viral clearance obtained in vitro with autologous T cells expanded by inactivated-virus-pulsed DC opens the possibility of an in vivo restoration of anti-HIV immunity, which is readily developed in most cases shortly after infection (probably before virus dissemination into lymph notes) (12) but is progressively lost during the course of the infection (20, 26).

APC functions (including up-regulation of T-cell proliferation, CTL response, and anti-HIV activities) of patient DC are enhanced by a nonantiviral concentration of PI (indinavir). This is no longer surprising, since recent in vivo and in vitro studies by our group (18, 19) and others (1, 28) show that PIs exhibit direct up-regulatory effects on proliferation and down-regulatory effects on apoptosis of patient T cells following immune stimulation. Thus, a PI (at both antiviral and nonantiviral concentrations) could be used as a potent adjuvant for optimizing the virus-specific CTL response in individuals following either preventive or therapeutic vaccination.

Although the in vivo evolving HIV-1 variants that evade the antiviral immunity developed during early infection have been known for many years (23), the reason that the infected host fails to mount de novo mutant-virus-specific immunity remains unknown. In a chronically HIV-infected individual (i.e., one in whom the virus has already been disseminated into lymph notes), viral replication is directly linked to local activation of lymphoid tissues characterized by huge in situ expression and release of cytokines. Certain components of these lymphoid cytokines (such as IL-10 and IFN-β) are known to interfere with generation of immature DC, and others (such as IL-1β, IL-6, TNF-α, IFN-α, and IFN-γ, etc.) provoke DC maturation.

Since supermatured DC lose their ability to process and present viral antigens (Fig. 4), it is conceivable that supermatured DC in immune-activated lymphoid tissues could not exert their APC function to process and present the evolving mutant antigens of viral variants. Such paralyzed DC in situ, in fact, could thus provide the prerequisite for establishing chronic HIV infection. However, our data demonstrate that such a defect in the generation of functional DC in HIV-infected patients can be overcome by DC-based vaccines generated in vitro from peripheral blood monocytes taken from infected patients.

It is interesting to observe that proviral DNA of patient PBMC can disappear (or become undetectable) when cocultured with autologous T cells pretreated with virus-pulsed DC with PI, suggesting that latent forms of HIV provirus might be rare in immune-activated lymphoid tissues. Our data suggest the possibility of eradicating the virus in vivo with a repeated vaccination regimen. Although HIV provirus might reside in quiescent T cells as a temporary viral reservoir escaping from recognition or killing by virus-specific effector cells (30), immune stimulation strategies such as IL-2-based therapy could help to activate quiescent T cells harboring HIV provirus, thereby exhausting such a temporary reservoir (3).

Taking these results together, it is clear that the complete eradication of HIV from the body of an infected individual represents a challenge. Reconstitution of anti-HIV immunity by potent DC-based therapeutic vaccines could be one of the keys to eventually curing HIV diseases. Controlled clinical trials are required to prove the utility of such an exciting and encouraging perspective.

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